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# Arvanil-induced inhibition of spasticity and persistent pain: evidence for therapeutic sites of action different from the vanilloid VR1 receptor and cannabinoid $CB_1/CB_2$ receptors

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**Abstract**

Activation of cannabinoid receptors causes inhibition of spasticity, in a mouse model of multiple sclerosis, and of persistent pain, in the rat formalin test. The endocannabinoid anandamide inhibits spasticity and persistent pain. It not only binds to cannabinoid receptors but is also a full agonist at vanilloid receptors of type 1 (VR1). We found here that vanilloid VR1 receptor agonists (capsaicin and *N-N′* -(3-methoxy-4-aminoethoxy-benzyl)-(4-*tert*-butyl-benzyl)-urea [SDZ-249-665]) exhibit a small, albeit significant, inhibition of spasticity that can be attenuated by the vanilloid VR1 receptor antagonist, capsazepine. Arvanil, a structural ''hybrid'' between capsaicin and anandamide, was a potent inhibitor of spasticity at doses (e.g. 0.01 mg/kg i.v.) where capsaicin and cannabinoid $CB_1$ receptor agonists were ineffective. The anti-spastic effect of arvanil was unchanged in cannabinoid $CB_1$ receptor gene-deficient mice or in wildtype mice in the presence of both cannabinoid and vanilloid receptor antagonists. Likewise, arvanil (0.1–0.25 mg/kg) exhibited a potent analgesic effect in the formalin test, which was not reversed by cannabinoid and vanilloid receptor antagonists. These findings suggest that activation by arvanil of sites of action different from cannabinoid $CB_1/CB_2$ receptors and vanilloid VR1 receptors leads to anti-spastic/analgesic effects that might be exploited therapeutically. 

*Keywords:* Cannabinoid; Vanilloid; Vanilloid VR1 receptor; Anandamide; Arvanil

## 1. Introduction

Pain and spasticity are common symptoms in multiple sclerosis and other conditions such as spinal injury, for which there is a paucity of effective therapies. Novel therapies for the alleviation of these symptoms, an area of therapeutic need, may be based on recent developments in the pharmacology of vanilloid and cannabinoid receptors.

The pungent ingredient of chili peppers, capsaicin, exerts its effects via the vanilloid receptor type 1 (VR1). This is a heat- and proton-gated, nonselective cation ion channel expressed in primary afferent nociceptive neurons (Caterina et al., 1997; Nagy and Rang, 1999). Persistent activation of vanilloid VR1 receptor results in de-sensitization of nociceptors and analgesia. There is evidence that bladder hyperreflexia following inflammation or spinal cord disease is attenuated by capsaicin. Furthermore, clinical intra-vesical therapy with capsaicin is somewhat successful in alleviating bladder symptoms in patients with both multiple sclerosis and spinal cord injury (De Seze et al., 1999), despite the invasive

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route of application and the stimulant nature of capsaicin. However, the effect of capsaicin on spasticity and tremors in animal models of multiple sclerosis has never been evaluated. Systemic capsaicin also demonstrates analgesic properties, attenuated by the vanilloid receptor antagonist, capsazepine, in both acute and tonic pain models (Perkins and Campbell, 1992; Urban et al., 2000). There is clinical evidence that topical capsaicin is effective for the treatment of chronic pain, including neuropathic pain (McQuay and Moore, 2000), but again, the stimulant side-effects limit its usefulness (Sindrup and Jensen, 1999). Nonpungent systemically administered capsaicin analogues represent a significant therapeutic advance in this field (Urban et al., 2000; Jaggar et al., 2001). In animal models, vanilloid receptor agonists ameliorate pain and bladder instability, two signs that can be also attenuated by cannabinoids (Jaggar et al., 1998; Farquhar-Smith and Rice, 2001).

So far, two cannabinoid receptors ($CB_1$ and $CB_2$) have been cloned. Endogenous ligands at cannabinoid receptors, including the prototypical ligand anandamide, have been identified, and pathways for their synthesis and inactivation (via cellular uptake and enzymatic hydrolysis) described (Di Marzo et al., 1998b). Both endogenous and exogenous cannabinoids are effective in relieving acute, tonic, neuropathic pain and bladder hyper-reflexia in various animal models (see Pertwee, 2001; Rice, 2001 for full reviews). The cannabinoid $CB_1$ receptor is expressed by neurons of the central and peripheral nervous system and plays a role in down-regulating the nerve growth factor component of inflammatory hyperalgesia and bladder hyper-reflexia (Rice, 2001). Recently, it has been reported that anandamide exerts agonist activity at both the cannabinoid $CB_1$ and vanilloid VR1 receptors (Zygmunt et al., 1999; Smart et al., 2000). In addition, synthetic vanilloid receptor agonists, such as olvanil, can interact with cannabinoid $CB_1$ cannabinoid receptors and the anandamide membrane transporter, which facilitates anandamide cellular uptake (Di Marzo et al., 1998a). Metabolically stable, nonpungent anandamide–capsaicin ''hybrids'' have been synthesized (Melck et al., 1999) and one of these analogues, arvanil ($\Delta^{5,8,11,14}$-*cis* eicosatetraenoyl-*N*-acyl-vanillyl-amide) behaves as a ''hybrid'' ligand at both cannabinoid $CB_1$ (but not $CB_2$) and vanilloid VR1 receptors. Arvanil also acts as a potent inhibitor of the anandamide transporter (Melck et al., 1999) and, thus, is in principle capable of activating cannabinoid $CB_1$ receptors indirectly by enhancing the levels of anandamide. In vitro evidence confirms that arvanil and anandamide have similar affinities for the cannabinoid $CB_1$ receptor (Di Marzo et al., 2000a), and that arvanil potently activates the vanilloid VR1 receptor (De Petrocellis et al., 2000; Ross et al., 2001). In vivo systemically administered arvanil is 100 times more potent than anandamide in the mouse ''tetrad'' of cannabinoid effects, consisting of (i) inhibition of spontaneous activity in an open field, (ii) induction of catalepsy on a ring, (iii) anti-nociception in the ''tail-flick'' assay, and (iv) rectal hypothermia. Although a positive response in all four tests is highly suggestive of cannabimimetic activity (Martin et al., 1991), the activity of arvanil in the mouse ''tetrad'' was not antagonized by the cannabinoid $CB_1$ receptor antagonist, *N*-(piperidin-1-yl-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride (SR141716A) (Di Marzo et al., 2000a). Based on these and other findings, it has been proposed that arvanil acts in central and extra-cerebral (i.e. spinal cord) nervous systems via non-cannabinoid $CB_1$, non-vanilloid VR1 receptors.

It has been demonstrated that cannabinoid receptor agonists, largely via $CB_1$, inhibit spasticity in multiple sclerosis model, chronic relapsing experimental autoimmune encephalomyelitis model in mice (Baker et al., 2000). Furthermore, enhancement of endocannabinoid levels through inhibition of endocannabinoid degradation also leads to amelioration of spasticity (Baker et al., 2001). Thus, if activation of vanilloid VR1 receptor also leads to anti-spastic actions, an agent that targets both the vanilloid and cannabinoid receptors, such as arvanil, may provide a significant therapeutic advance for the treatment of both pain and spasticity. Therefore, in this study, we have first investigated whether vanilloid VR1 receptor activation alleviates spasticity in a mouse model of multiple sclerosis and then evaluated the potential anti-spastic properties of arvanil in this model, as well as its analgesic activity in the rat formalin test of persistent pain.

## 2. Materials and methods

### 2.1. Chemicals

Capsaicin, capsazepine and (*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone (WIN 55,212) were purchased from RBI/Sigma (Poole, UK). Methanandamide was purchased from Tocris (Bristol, UK). SR141716A and *N*-[(1*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]5-(4-choro-3 methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide (SR144528) were a gift from the National Institute of Drug Abuse and the National Institute of Mental Health chemical synthesis program (Bethesda, USA). *N*-*N*′-(3-methoxy-4-aminoethoxy-benzyl)-(4-tert-butyl-benzyl)-urea (SDZ-249-665), a nonpungent vanilloid VR1 receptor agonist, was provided by Novartis (London, UK) (Urban et al., 2000). Arvanil was synthesized as described previously (Melck et al., 1999) or was obtained from Cayman chemicals (Ann Arbor, USA).

### 2.2. Animals

Biozzi ABH mice were from stock bred at the Institute of Neurology. CD1 wildtype and CD1 $CB_1$ gene (*Cnr1*) disrupted ($CB_1$.*Cnr1* − / − ) mice (Ledent et al., 1999) were obtained from Charles Rivers (UK). CD1 mice are outbred and have low experimental autoimmune encephalomyelitis

(EAE) susceptibility (CD1.*Cnr1*+/+ wildtype 0/5 EAE susceptible, CD1.*Cnr1* − / − 1/6 EAE) compared to ABH. Disease in ABH mice is dominant (Baker et al., 1995) and ABH mice were outcrossed with CD1. *Cnr1* − /+ were backcrossed with ABH mice twice and intercrossed to produce homozygous mice (ABH.*Cnr1* − / − ). Tail DNA samples were screened by PCR (cycles (30 ×): 94° 30 s, 55° 60 s, 72° 30 s). Using *Cnr1* (5′ CAT CAT CAC AGA TTT CTA TGT AC 3′ and GAG GTG CCA GGA GGG AAC C) and targeting construct (5′ GAT CCA GAA CAT

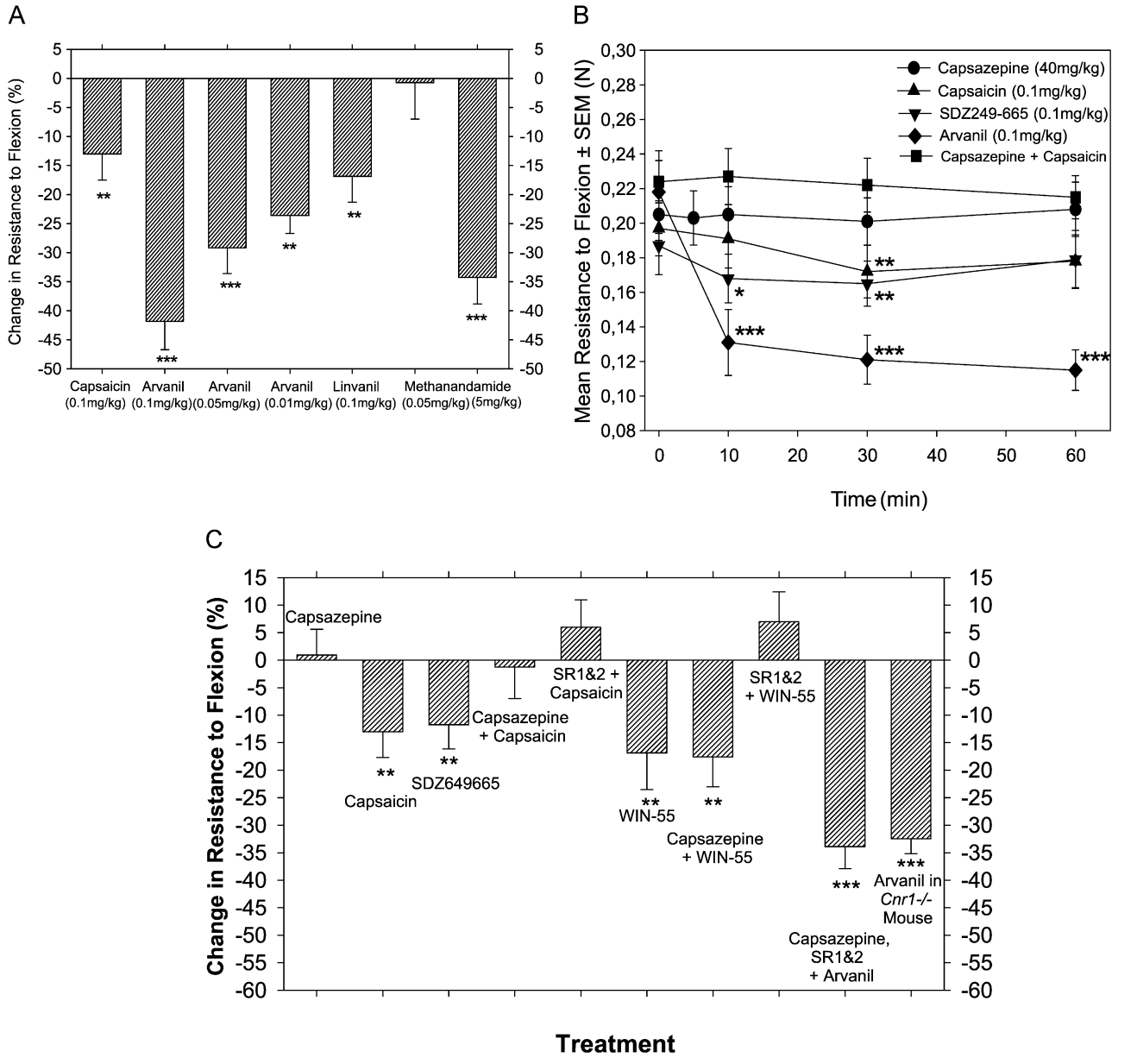


Fig. 1. Spasticity is limited by arvanil and by VR1 and CB receptor-mediated effects. Following the development of spasticity occurring after induction of chronic relapsing experimental autoimmune encephalomyelitis, the forces required to bend individual hind limbs to full flexion against a strain gauge were assessed (>6 mice/group) before and following injection i.v. with arvanil, linvanil, capsaicin and methanandamide at the doses indicated. The results represent (A) the mean ± S.E.M. change in resistance to flexion 30 min after injection compared to baseline levels (0%) and (B) the mean ± S.E.M. resistance to flexion. (C) Following the development of spasticity, resistance to flexion was measured before and following injection i.v. with capsaicin (0.1 mg mg/kg), SDZ-249-665 (0.1 mg/kg) or *R*(+)WIN 55,212 (1mg/kg), or in ABH.*Cnr1* − / − mice following injection i.v. with arvanil (0.05 mg/kg). The results represent the mean ± S.E.M. change in resistance to flexion 30 min after injection compared to baseline levels (0%) at 0 min. In some instances, animals were pretreated ( − 20 min) previously with capsazepine (40 mg/kg i.v.) or SR141716A and SR144528 (SR1 and SR2 5mg/kg i.v.), or both. Vehicle administration yielded a response similar to that of capsazepine alone (not shown). The two antagonists administered alone induce a transient worsening of spasticity, which had disappeared before the agonists were administered (Baker et al., 2000). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with baseline values by ANOVA followed by the Student–Newman–Keuls test.

CAG GTA GG 3′ and 5′ AAG GAA GGG TGA GAA CAG AG 3′) specific primers and *Cnr1* flanking microsatellites (http://www.informatics.jax.org., Research Genetics, Huntsville, USA; Baker et al., 1995). These were shown to have a functional deletion by their ability to tolerate 10–20 mg/kg *R*(+)WIN 55,212 i.p. without sedation. Male Wistar rats (200–250 g) were purchased from Bantin and Kingman (Hull, UK), housed in colony cages and maintained on a 12-h light/dark cycle. All experiments were approved by the United Kingdom Home Office.

### 2.3. Induction of spasticity

Induction of chronic relapsing experimental autoimmune encephalomyelitis following injection of spinal cord homogenate in Freund's adjuvant in 6–8-week-old mice and assessment of spasticity against a strain gauge was as described previously (Baker et al., 2000, 2001). Animals were injected either intravenously (i.v.) or intraperitoneally (i.p.) with compounds in either Tween 80/PBS (Baker et al., 2000) or intralipid® 30% (Pharmacia Laboratories, Milton Keynes, UK). Results were compared (pairwise) using one-way repeated measures analysis of variance (Student–Newman–Keuls method) using SigmaStat v2.0 (Jandel, USA).

### 2.4. Assessment of hypothermia and inhibition of locomotor activity

Temperature was monitored momentarily before and following injection of compounds using a thermocouple thermometer (Portec P9005 thermometer, Wrestlington, UK). The thermocouple was held firmly onto the abdomen by the covering hind limb. The temperature was allowed to equilibrate at its maximum before recording activity in a 27 × 27-cm open-field activity monitor (Med Associates, Georgia, VT, USA). Activity was assessed over an 8-min period.

### 2.5. Assessment of persistent pain

The formalin test is a well-established model of persistent somatic pain (Dubuisson and Dennis, 1977) that has been lately refined (Tjolsen et al., 1992; Watson et al., 1997). The animals were acclimatized to the testing environment (clear Plexiglas box 23 × 18 × 14 cm) for 15 min and allocated into one of six treatment groups, in which drugs were administered intraperitoneally (i.p.). The groups were; solvent control (1:19 dimethylsulfoxide [DMSO]/saline), 0.1 or 0.25 mg/kg arvanil or 0.25 mg/kg arvanil with prior administration of

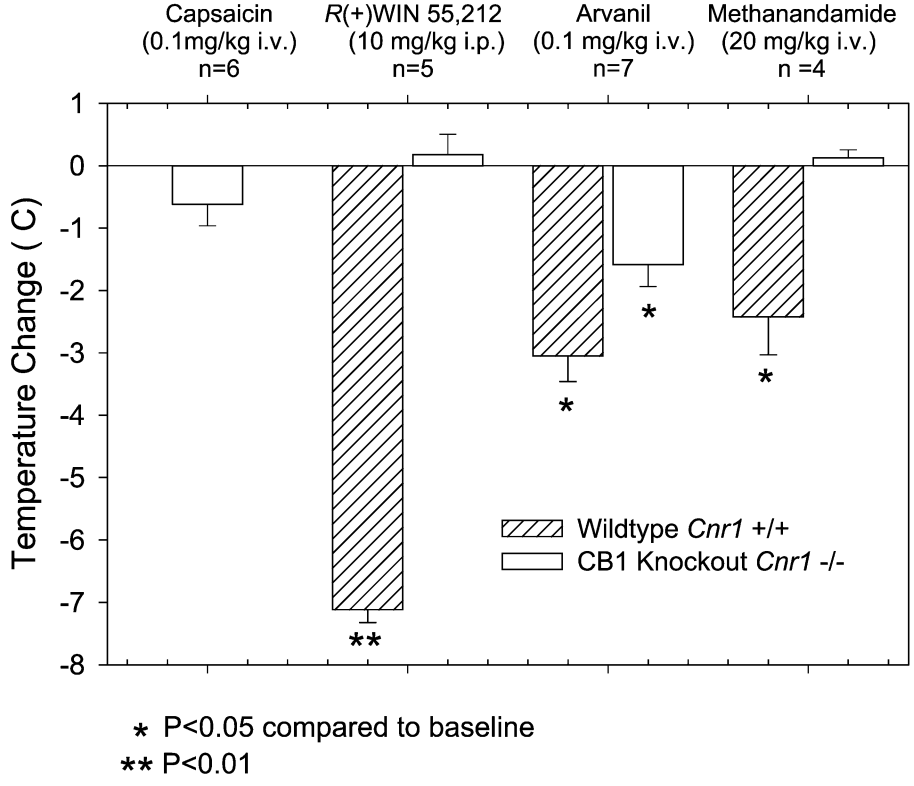


Fig. 2. Hypothermia induced by cannabinoids in $CB_1$ gene-deleted mice. Mice either wildtype (*Cnr1*+/+) or knockout (*Cnr1* − / − ) were injected with arvanil, capsaicin, methanandamide or *R*(+)WIN 55,212 at the doses indicated. Temperatures (°C) were measured via a thermocouple placed under the hind limb. The results represent the mean ± S.E.M. ($n = 4$) temperature change 20 min after drug administration. Vehicle administration yields a nonstatistically significant response (not shown). Data for each chemical were obtained in different experiments. Baseline temperatures ranged between 36 and 37 °C. At baseline, the mean temperature ± S.D. of vehicle-treated wildtype mice was 36.7 ± 0.3 °C ($n = 7$) and 36.9 ± 0.4 °C ($n = 7$) in vehicle-treated *Cnr1* − / − mice.

either 0.5 mg/kg SR141716A, 1 mg/kg SR144528, or 3 mg/kg capsazepine (all administered in 1:19 DMSO/saline solvent, except SR144528 dissolved in 40% DMSO). Thirty minutes later, 50 μl of 2.5% formalin were injected subcutaneously into the dorsal surface of the right hind paw, and the total time spent in two distinct behavioral categories ((a) lifting the paw or (b) licking, biting or shaking the paw) for each 5-min period over 50 min recorded. The animals were sacrificed at the end of the experiment. Nociceptive behavior was quantified using the composite pain score-weighted scores technique (CPS-$WST_{0,1,2,3}$) (Watson et al., 1997), where behavior (a) weighted times one and behavior (b) weighted times two. An overall CPS-$WST_{0,1,2}$ was calculated for the first 0–15-min and the second 15–50-min phases of the response. The overall CPS-$WST_{0,1,2}$ for the first and second phases of the behavioral response were compared to the control value using one-way analysis of variance (Dunnett's method), with $P < 0.05$ set as the level of significance (SigmaStat v2.0, Jandel).

## 3. Results

### 3.1. Spasticity

The maximum tolerated dose of capsaicin (0.1 mg/kg i.v.) induced a modest but nevertheless significant reduction ($P < 0.01$) in the degree of spasticity (Fig. 1A,B,C), peaking at approximately 30 min after injection (Fig. 1B). Prior administration (10–20 min) of the vanilloid VR1 receptor antagonist, capsazepine (40 mg/kg), prevented the anti-spastic effect of capsaicin (Fig. 1B and C). A similar inhibition of spasticity was also detected following the injection (0.1 mg/kg i.v.) of the nonpungent vanilloid VR1 receptor agonist, SDZ-249-665 (0.1 mg/kg i.v.). Following the injection of a combination of SR141716A (a cannabinoid $CB_1$ receptor selective antagonist, 5 mg/kg i.v.) and SR144528 (a cannabinoid $CB_2$ receptor selective antagonist, 5 mg/kg i.v.), tail and hind limb spasticity increased, although this effect rapidly returned ($P > 0.05$) to starting levels (Baker et al., 2000) within 20 min. Although the enhanced spasticity following cannabinoid receptor antagonism was short-lasting, the antagonist effect on the cannabinoid $CB_1$/$CB_2$ receptor agonist, *R*(+)-WIN-55,212, was maintained for at least 2 h following administration of the agonist, as indicated also by an inhibition of the sedative effect and hypothermia of *R*(+)-WIN-55,212 (10 mg/kg i.p.) in normal animals (Fig. 2). In contrast, no enhancement or reduction of signs was evident following injection of the vanilloid receptor antagonist, capsazepine (Fig. 1B), which thus behaves as a vehicle control (Baker et al., 2000). Pretreatment with SR141716A (5 mg/kg i.v.) and SR144528 (5 mg/kg i.v.) also completely eliminated the anti-spastic effect of vanilloid VR1 receptor agonism by capsaicin (Fig. 1C). The anti-spastic effect of *R*(+)-WIN-55,212 (1 mg/kg i.p.), was eliminated with a combination of SR141716A (5 mg/kg) and SR144528 (5 mg/kg) pre-administration, but not with capsazepine (40 mg/kg) pre-treatment (Fig. 1C).

Arvanil induced a rapid amelioration of the spastic response, assessed by resistance to flexion of the hind limb, when injected intravenously at either 0.1 mg/kg ($P < 0.001$), 0.05 mg/kg ($P < 0.001$) and 0.01 mg/kg ($P < 0.01$) (Fig. 1A and B). The maximal response was obtained within 10–30 min following injection (Fig. 1B). An arvanil analogue, linvanil (Melck et al., 1999), was less efficacious. Arvanil efficacy was comparable to or better than that of the cannabinoid $CB_1$ receptor agonist methanandamide (5.0 mg/kg, $P < 0.001$, Fig. 1A) despite the fact that much lower doses were injected. Interestingly when vanilloid VR1 and cannabinoid receptor antagonists were both administered prior to arvanil, injected at a dose (0.05 mg/kg) that did not induce hypothermia, there was still a significant inhibition ($P < 0.001$) of resistance to flexion (Fig. 1C), comparable to that observed in animals injected with arvanil alone (Fig. 1A).

At 0.1 mg/kg i.v., arvanil induces cannabinoid "tetrad" effects including hypothermia (Fig. 2) and hypomotility (Di Marzo et al., 2000a). These effects were also apparent in $CB_1$ gene (*Cnr1*)-deleted CD1 mice, where there was a significant decrease in thermoregulation (Fig. 2) and locomotor activity ($93 \pm 8\%$ decrease, $n = 4$). By contrast, the hypothermic effects of methanandamide (20 mg/kg i.v.) and

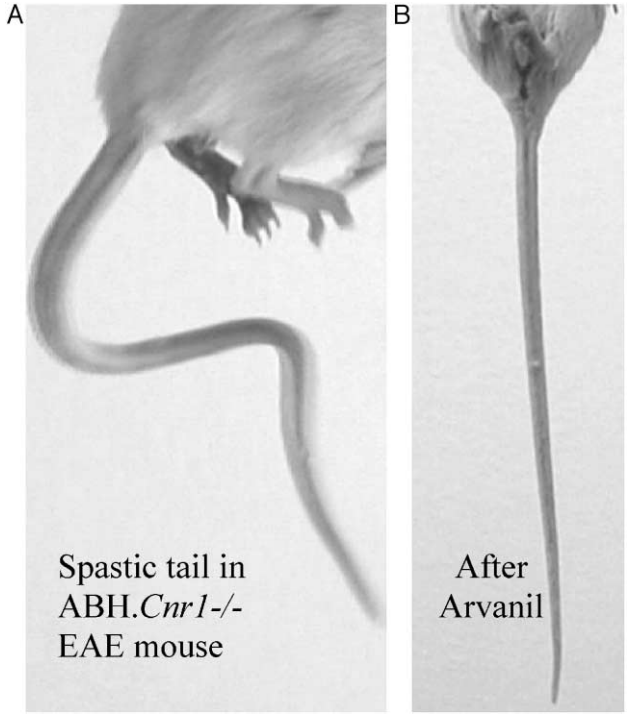


Fig. 3. Spasticity is limited by arvanil in the absence of $CB_1$. Spasticity develops in ABH.*Cnr1* −/− mice after CREAE induction. Although disease is typically associated with paresis of the tail, this can become spastic and show atypical (A) tail twisting (Baker et al., 2000, 2001). This was (B) inhibited following injection of arvanil (0.05 mg/kg i.v.).

*R*(+) WIN-55,212, and other obvious cannabimimetic sedative effects were inhibited in *Cnr1* − / − mice, although locomotor activity still appeared strongly impaired (data not shown), in agreement with a recently reported study suggesting for both anandamide and *R*(+) WIN-55,212 the existence of additional cannabinoid receptors in *Cnr1* − / − mice (Breivogel et al., 2001). These observations further suggest the existence of non-cannabinoid $CB_1$ receptors that might also mediate the anti-spasticity effect of arvanil since we found that chronic relapsing experimental autoimmune encephalomyelitis-induced spasticity in ABH.*Cnr1* − / − mice still responded positively to arvanil (0.05 mg/kg) treatment (Figs. 1C and 3).

### 3.2. Pain

The control group confirmed the previously described characteristic biphasic response (Watson et al., 1997). Both

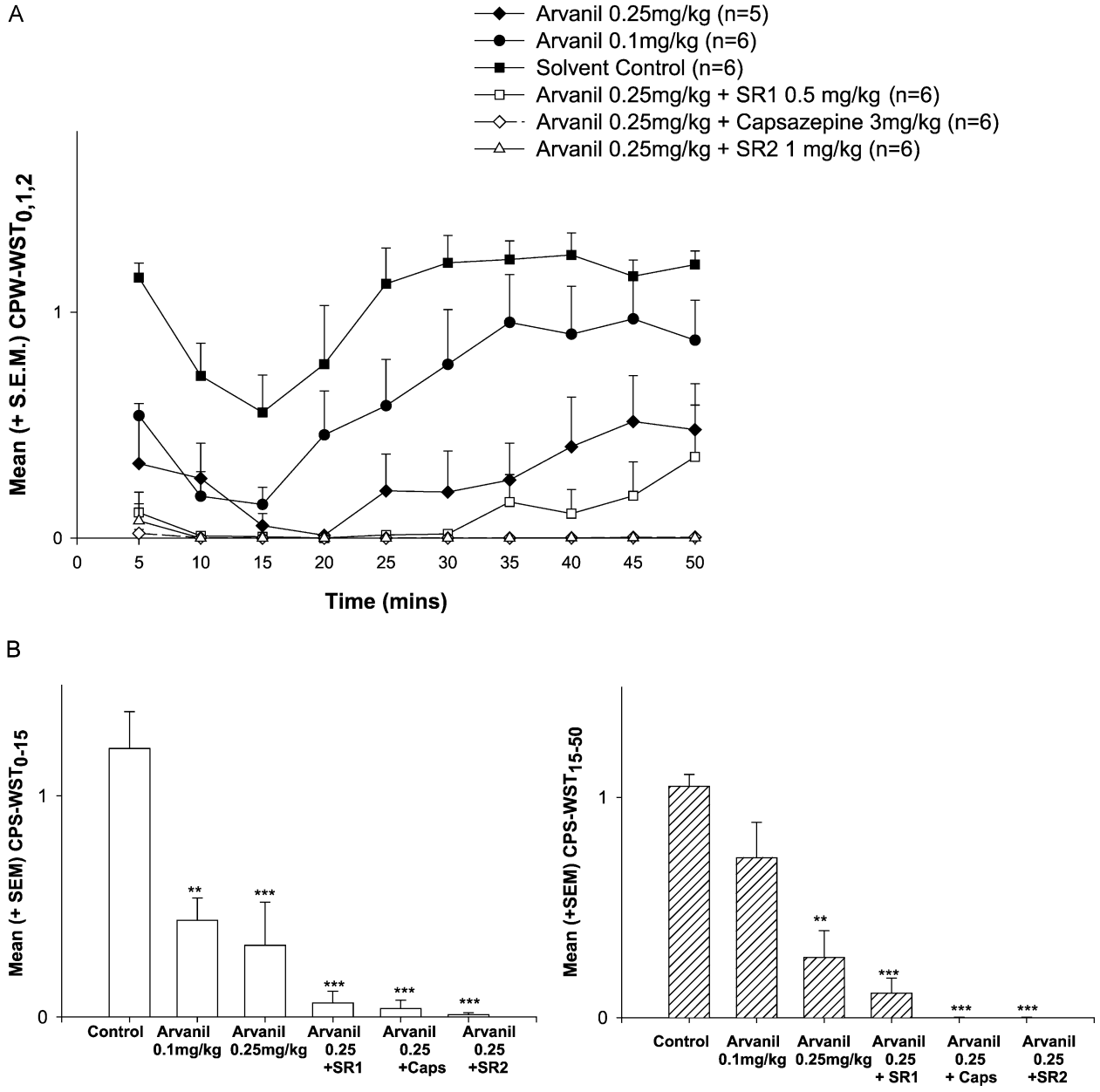


Fig. 4. Pain behavior in the formalin test is attenuated by arvanil. Time course (A) of nociceptive activity (CPS-$WST_{0,1,2}$ + S.E.M.) for the six treatment groups (0.1 or 0.25 mg/kg arvanil or 0.25 mg/kg arvanil with prior administration of either 0.5 mg/kg SR141716A [SR1], 1 mg/kg SR144528 [SR2], or 3 mg/kg capsazepine [caps]) following s.c. 2.5% formalin in the dorsal hind paw. Mean pain scores (B) for both the early (0–15 min in grey) and late phases (15–50 min in stripes) of the formalin response. SR1 and SR2 alone induce a weak anti-nociceptive response in this animal model (Beaulieu et al., 2000), whereas capsazepine alone was significantly active in the first phase and exhibited a nonstatistically significant trend for anti-nociception in the second phase (data not shown). ** $P<0.01$, *** $P<0.001$ compared with control values by ANOVA (followed by the Dunnett's test).

phases of the CPS-$WST_{0,1,2}$ were inhibited ($P < 0.05$) dose-dependently by arvanil, although the observed reduction with 0.1 mg/kg in the second phase did not reach statistical significance ($P > 0.05$). Neither 0.5 mg/kg SR141716A, 1 mg/kg SR144528 nor 3 mg/kg capsazepine reversed the analgesic activity of 0.25 mg/kg arvanil (Fig. 4). In fact, the antagonists appeared instead to potentiate the analgesic effects of arvanil, very probably because they are capable of inducing per se a weak anti-nociceptive response (significant only for the first phase in the case of capsazepine) in the same pain model (Beaulieu et al., 2000, and data not shown).

## 4. Discussion

In this study, we have shown that activation of native vanilloid receptors alleviates spasticity in the chronic relapsing experimental autoimmune encephalomyelitis mouse model of multiple sclerosis, and that arvanil, a cannabinoid $CB_1$/vanilloid VR1 receptor ''hybrid'' agonist, exhibits potent inhibitory activity in this model in addition to potent analgesic activity in the rat formalin paradigm of persistent pain. However, these two actions of arvanil appear to be due largely to mechanisms that are not mediated by cannabinoid $CB_1$/$CB_2$ and vanilloid VR1 receptors.

### *4.1. Spasticity in CREAE mice*

Spasticity develops following the accumulation of neurological deficits induced by autoimmune attack of the central nervous system (CNS) during chronic relapsing experimental autoimmune encephalomyelitis (Baker et al., 2000). We have previously shown that activation of cannabinoid receptors (which are widely distributed in both central and peripheral sensory neurons) and inhibition of endocannabinoid inactivation via cellular uptake and enzymatic hydrolysis ameliorate spasticity in mice with chronic relapsing experimental autoimmune encephalomyelitis (Baker et al., 2000, 2001). We have suggested that endocannabinoids produced during spasticity exert a tonic beneficial action against spasticity in this animal model of multiple sclerosis. In agreement with the presence of vanilloid VR1 receptors in dorsal root ganglia, spinal cord and brain (Szallasi and Di Marzo, 2000), we now report that two vanilloid VR1 receptor agonists (capsaicin and SDZ-249-665) also have a significant, albeit modest, anti-spastic effect, which is inhibited by capsazepine, thus indicating that these compounds may have a potential role in the treatment of spasticity. Several possible explanations for this effect can be put forward, such as, for example, vanilloid VR1 receptor-mediated depletion of excitatory neurotransmitters (e.g. glutamate) from dorsal root ganglia and spinal neurons. Another possibility is that vanilloid VR1 receptor activation in spinal neurons leads to increased formation of endocannabinoids with anti-spastic activity (see below). At any rate, in mice with chronic relapsing experimental autoimmune encephalomyelitis, vanilloid VR1 receptor antagonism with capsazepine alone failed to affect the basal level of spasticity. This finding is in contrast to the rapid increase in spasticity following cannabinoid receptor antagonism (Baker et al., 2000, 2001), and suggests that an endovanilloid tone does not influence spasticity. In view of the anti-spastic effect of VR1 agonists, the previously reported (Baker et al., 2001) similar action by AM404, an inhibitor of endocannabinoid cellular uptake with agonist activity at vanilloid VR1 receptors, can be re-interpreted as the sum of two distinct effects, one exerted via VR1 receptors and the other through inhibition of endocannabinoid uptake. This would explain why AM404, although exhibiting vanilloid VR1 receptor agonist activity lower than that of capsaicin, and anandamide transporter inhibitory activity comparable to that of the more selective inhibitor VDM11 (De Petrocellis et al., 2000), produces a significantly more efficacious anti-spastic response than either capsaicin (this study) or VDM11 (Baker et al., 2001).

As expected, although CB antagonism could inhibit the anti-spastic effect of the cannabinoid $CB_1$ receptor agonist *R*(+)-WIN-55,212, there was no readily apparent inhibition of this response when the same animals had been pretreated with capsazepine. In contrast, pretreatment with CB antagonists eliminated the anti-spastic effect of vanilloid VR1 receptor agonism by capsaicin. The capacity of SR141716A to inhibit VR1-mediated effects of capsaicin has been reported previously (Zygmunt et al., 1999). This might suggest a link between the vanilloid and cannabinoid receptor system, e.g. a vanilloid receptor effect upstream of the cannabinoid receptor system. One possibility is that VR1 agonism promotes the release of endocannabinoids, as shown in vitro by using capsaicin and cells over-expressing rat VR1 (Di Marzo et al., 2001). Alternatively, there could be cross-talk during downstream receptor signaling events. Finally, it has been indicated recently that, at high doses, SR141716A can antagonize the human vanilloid VR1 receptor in vitro (De Petrocellis et al., 2001). Therefore, it is possible that the effect observed here with this cannabinoid receptor antagonist was simply due to direct inhibition of VR1 rather than through an indirect effect via cannabinoid receptors.

Once that vanilloid receptor activation was established to exert a weak although significant beneficial effect against spasticity in chronic relapsing experimental autoimmune encephalomyelitis mice, we reasoned that a compound capable of activating at the same time both cannabinoid $CB_1$ and vanilloid VR1 receptors would have exhibited an even higher anti-spastic activity than ''pure'' agonists of these two receptor classes. Indeed, arvanil was extremely potent in inhibiting spasticity in chronic relapsing experimental autoimmune encephalomyelitis mice. Arvanil ''hybrid'' action may in principle explain this very efficacious anti-spastic effect, which lasted for over 30 min and could be observed with doses (i.e. 0.01 mg/kg) well below those inducing undesirable ''central'' effects. Yet, arvanil has a lower affin-

ity/agonist activity at cannabinoid $CB_1$ receptors in vitro compared with methanandamide (Pertwee, 1999), which at a comparable low dose failed to inhibit spasticity within this time frame (Baker et al., 2000). Linvanil, an arvanil homologue with similar activity on the anandamide membrane transporter, 10-fold lower activity on VR1 receptors, and almost no affinity for cannabinoid $CB_1$ receptors (Melck et al., 1999; De Petrocellis et al., 2000), inhibited spasticity less potently than arvanil, but at much lower doses than AM404, which is as active on the transporter but 10-fold less potent at VR1 (De Petrocellis et al., 2000). Finally, and most importantly, arvanil anti-spastic effect was still observed in the presence of cannabinoid receptor antagonists or in *Cnr1* −/− chronic relapsing experimental autoimmune encephalomyelitis mice. Therefore, it is possible that stimulation of VR1, rather than direct or indirect (via inhibition of endocannabinoid degradation) activation of $CB_1$ receptors, accounts for arvanil's very potent anti-spastic activity. However, when capsazepine, at a dose fully effective against capsaicin, was administered prior to arvanil, there was still a strong inhibition of resistance to flexion comparable to that observed in animals injected with arvanil alone. These data suggest that the major anti-spastic effect of arvanil is via a non-$CB_1$, non-$CB_2$, non-VR1 site of action. However, it must be noted that, although capable of inhibiting the anti-spastic effect observed with capsaicin, capsazepine, the only readily available VR1 antagonist to date, is considered to be a weak antagonist in vivo. As arvanil is more active at vanilloid VR1 receptors than capsaicin (Ross et al., 2001; De Petrocellis et al., 2000), it is also possible that capsazepine is not entirely efficacious in inhibiting the VR1-mediated activity of arvanil in vivo. Studies on VR1 knockout mice with chronic relapsing experimental autoimmune encephalomyelitis will be necessary to rule out the participation of vanilloid VR1 receptors in arvanil anti-spastic activity.

### 4.2. Persistent pain

In the rat formalin model of somatic pain, the first phase is due to direct stimulation of nociceptors by formalin, whereas the second phase has been considered due to peripheral and central sensitization (Dickenson and Sullivan, 1987; Tjolsen et al., 1992). Arvanil at 0.25 mg/kg potently attenuated both phases of the formalin pain response, compared with a less potent inhibition of the second phase with 5 mg/kg of anandamide (Jaggar et al., 1998). It can be thus estimated that arvanil is at least 20-fold more potent than anandamide in this assay of persistent pain. The effect of arvanil was not reversed by prior administration of cannabinoid $CB_1$/$CB_2$ receptor antagonists at doses comparable with those that are effective against anandamide and palmitoylethanolamide in other pain models (Farquhar-Smith and Rice, 2001), nor by capsazepine. The vanilloid receptor antagonist could not be tested against capsaicin since the latter compound causes a strong nociceptive response when administered under similar conditions, and requires a several hour pretreatment to induce instead an anti-nociceptive response, mostly due to desensitization/death of nociceptive neurons (Dray and Dickenson, 1991). Therefore, the data in this rat pain model suggest the lack of involvement of $CB_1$, $CB_2$ and VR1 receptors in arvanil analgesic effect and corresponds with the potent and capsazepine-insensitive anti-nociceptive activity in the mouse tail flick acute pain model (Di Marzo et al., 2000a), lending support to the hypothesis of a novel site of action for arvanil.

### 4.3. Non-$CB_1$, non-VR1 molecular targets for arvanil?

Although it is believed that the majority of the psychotropic effects of $\Delta^9$-tetrahydrocannabinol, such as, for example, those in the mouse "tetrad" of tests, are mediated by cannabinoid $CB_1$ receptors, arvanil (0.2 mg/kg i.v.) has been shown to induce cannabimimetic activity in these tests in the presence of $CB_1$ antagonism, suggesting further evidence for a third cannabinoid receptor (Di Marzo et al., 2000b). It has been found here that arvanil, apart from still effectively reducing spasticity in ABH.*Cnr1* −/− mice with chronic relapsing experimental autoimmune encephalomyelitis, can also reduce core temperature and locomotor activity in *Cnr1* −/− mice, in agreement with previous findings showing that these effects were not counteracted by SR141716A (Di Marzo et al., 2000a). Several other studies further support the possible existence of non-$CB_1$, non-$CB_2$, non-VR1 molecular targets for arvanil and other anandamide-like molecules: (1) livanil, another arvanil homologue with almost no affinity for cannabinoid $CB_1$ receptors, when administered i.p. inhibits spontaneous activity in rats 10-fold more potently than either anandamide or capsaicin. The effect is not antagonized by either capsazepine or SR141716A (Di Marzo et al., 2001); (2) anandamide has been also found to have some cannabimimetic "tetrad" effects in $CB_1$ gene (*Cnr1*)-deleted C57BL/6 mice, possibly through a G-protein-coupled receptor (Di Marzo et al., 2000b; Breivogel et al., 2001). By contrast, the "tetrad" effects of $\Delta^9$-tetrahydrocannabinol were inhibited in the same transgenic mice (Di Marzo et al., 2000b). (3) Anandamide and *R*(+)WIN-55,212, but not other cannabinoid $CB_1$ receptor agonists, activate G-protein-coupled receptors in the brain of *Cnr1* −/− mice (Breivogel et al., 2001) and inhibit hippocampal excitatory neurotransmission in these transgenic mice (Hajos et al., 2001). (4) O-2093, an arvanil analogue with low affinity for the CB1 receptor and no efficacy at the VR1, is extremely efficacious and potent in all four parameters of the mouse "tetrad" (Di Marzo et al., 2002). These observations suggest that compounds based on the structure of anandamide may have selectivity for additional, possibly novel, sites of action, for which arvanil might have higher affinity and efficacy. A possible location of these proteins could be the spinal cord, which is involved in the control of nociceptive responses, and whose damage results in spasticity in chronic relapsing experimental autoimmune encephalomyelitis and multiple sclerosis.

The lack of specificity of the available pharmacological tools will require the use of *Vr1*, *Cnr1* and *Cnr2* triple gene-deleted mice to definitively demonstrate additional molecular targets for arvanil. Although it has been postulated that the cannabinoid system may be an important regulator of neurotransmitter function and that the $CB_1$ gene has been evolutionary conserved as a single-coding exon, *Cnr1*-deleted mice show no major deleterious phenotype, which may suggest a compensatory mechanism that is not provided by over-expression of cannabinoid $CB_2$ receptors in the CNS of *Cnr1*-disrupted mice (Zimmer et al., 1999). A putative arvanil-binding protein may provide such a compensatory mechanism, and, more importantly from a therapeutic point of view, would represent a target for the development of potent anti-spasticity and analgesic drugs.

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